IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re the Application of:	)	Group Art Unit: 1652
BERRY et al.	)	Examiner: Fronda, C.
Serial No.: 09/341,600	)	
Filed: September 15, 1999	)	DECLARATION OF DR. ARNOLD L. DEMAIN (Under 37 CFR 1.132)
Atty. File No.: 3161-18-PUS	)	
For: "PROCESS FOR PRODUCTION OF N-GLUCOSAMINE"	)	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313

Dear Sir:

I, Arnold L. Demain, declare as follows:

1. I am an expert in the field of fermentation biology and strain improvement, and I have worked in this field for more than 50 years. Since 2001, I have been a Research Fellow in Microbial Biochemistry at The Charles A. Dana Research Institute for Scientists Emeriti (R.I.S.E.) at Drew University in Madison, New Jersey. I am also Visiting Professor at Rutgers University. I was formerly Professor of Industrial Microbiology in the Department of Biology at the Massachusetts Institute of Technology (MIT) for 32 years. Prior to MIT, I was employed by Merck & Co., Inc., and was founder and head of the Department of Fermentation Microbiology. I received my Bachelor of Science and Master of Science degrees in bacteriology from Michigan State University in 1949 and 1950, respectively, and my doctorate in microbiology in 1954 from the University of California at Davis and Berkeley. My honors, awards, and honorary degrees are listed in my curriculum vitae, which is attached to this Declaration. I have published over 490 publications, co-authored or co-edited 11 books, and hold 21 U.S. patents. I am also a member of the National Academy of Sciences in the United States, Mexico, and Hungary, and a member of the Board of Governors of the American Academy of Microbiology. This summary

of my experience, in conjunction with the attached curriculum vitae, provides evidence of my extensive experience in the fields of fermentation biology and strain improvement.

2. I have been engaged as an independent third party expert by Arkion Life Science LLC d/b/a/ Bio-Technical Resources Division, of Wilmington, Delaware, which is the Assignee of the above-identified application, to review the file of the above-identified application, and to provide my comments on certain issues in the prosecution of the application. I was compensated for my time on an hourly basis.

3. This Declaration is being submitted in conjunction with an Amendment and Response to an Office Action having a mailing date of April 21, 2005 in the above-identified application.

4. I have reviewed, and I am familiar with, the above-identified application and the presently pending claims, and I have also reviewed several documents from the file history of this application, including the prior Declaration under 37 CFR 1.132 that was submitted by Dr. Ming-De Deng, and the Office Action mailed April 21, 2005. The following discussion provides my comments on the Examiner's rejection of Claims 40-76 under 35 U.S.C. § 112, first paragraph, on the basis of enablement. In particular, the following discussion addresses the Examiner's argument that the specification does not enable the invention that is claimed.

5. Comments on the Examiner's Rejection

Introduction.

The lifeblood of the fermentation industry is the ability to improve microbial strains genetically. This has been practiced since the nineteen forties at a time when penicillin was a wartime necessity. At first, it was done by random mutation, *i.e.*,"brute-force" **screening** technology in which the wild-type strain, and successive improved strains, were mutagenized by toxic chemicals (such as nitrosoguanidine) or physical agents (such as Xrays or UV). Most of the population was killed and the remaining cells were screened by fermentation in flasks containing a liquid medium for a number of days, and testing the resultant broths for the amount of product made. Such screening could involve as many as 10,000 to 100,000 surviving isolates before an improved strain was isolated.

This tedious procedure was improved over the years by more convenient screening methods in which product formation was first assayed on agar plates, *e.g.*, measuring the

diameter of a clear zone ("halo") around an antibiotic-producing colony or around an agar plug containing antibiotic-producing cells on its surface (the "agar-plug" method), or the color of the colony in the case of pigmented products, or the diameter of a colored halo around a colony producing a compound capable of a chemical reaction producing a color in the presence of a reagent, or the diameter of a cloudy halo around a colony producing a growth-promoting compound.

Another major improvement was the employment of **selection**, *i.e.*, the use of antimetabolites to kill a large part of the population surviving mutagenesis and thus decrease the number of isolates to be assayed by fermentation. In such cases, the antimetabolites were chosen on a rational basis, usually structurally-related analogs of the desired compound, to select those mutants which were most likely improved in production ability.

The molecular biology revolution occurring in the mid-1950's, and the biotechnology revolution of the 1970's allowed a more rational basis for creating genetic diversity than random mutation. This included the transfer of genes between species using plasmid transformation, transposon mutagenesis, protoplast fusion, homologous recombination, polymerase chain reaction (PCR) cloning, gene sequencing, DNA shuffling, whole genome shuffling and other techniques. However, such modern gene manipulation employed for strain improvement never eliminated the need for screening/selection. It merely cut down on the number of isolates needed to be screened from hundreds of thousands or tens of thousands to a few thousand or even less. Thus, screening/selection is an obligatory part of strain improvement, *i.e.*, it is a routine procedure used in various forms by all microbiologists, microbial geneticists, and molecular biologists. Recent improvements in screening technologies have involved miniaturization of flask fermentation into test tubes, or into microtiter plates containing 96 or more wells, followed by assay by high throughput screening techniques. Clearly, screening is still essential and routine to any molecular genetic improvement process.

Comments on Enablement Rejection and Claimed Invention.

I do not agree with the Examiner when he states that routine experimentation in the art does not include making vast numbers of mutants, and screening and selecting said mutants. I also do not agree when the Examiner states that one skilled in the art would require additional guidelines, such as the specific type of genetic modification to perform on the specific proteins

or enzymes of claimed microorganisms, or the amino acid residues which can be modified that lead to the claimed effect, or the gene encoding the enzyme and its biological source. The Examiner is mistaken when he states that without such guidance, the experimentation left to those skilled in the art is undue or is unpredictable. In my mind and experience, it is not undue or unpredictable. Indeed, it is an expected type of effort; it is simple, and will succeed as long as the method of screening/selection is clearly presented, as it has been in the above-identified patent specification. The Examiner is mistaken when he states that the work of the experimenter would include selecting from a variety of genetic modifications such as replacing the wild-type promoter of the glucosamine-6-phosphate synthase, modifying the nucleic acid sequence encoding the synthase, and selecting proteins other than the synthase to modify. Indeed, it is my view that these efforts would be unnecessary to carry out the invention described in the above-identified application.

I agree with the Applicants that the original patent specification describes the development of a strain by both classical strain development and molecular genetic techniques and gives a detailed description and working examples of how to use molecular genetic techniques and classical strain development. I also agree with the Applicants' contention that one skilled in the art could produce strains with increased glucosamine-6-phosphate synthase activity without the knowledge of where in the nucleotide sequence or enzyme sequence the mutation occurs. I further agree with the Applicants when they state that routine experimentation does include making a vast amount of mutants and screening and selecting those which have the desired phenotype of increased glucosamine-6-phosphate synthase activity. I am in agreement with the Applicants' contention that biologists have been producing and selecting desired mutants for many years, and that the predictability of producing an important strain using the guidance in the patent specification is quite high, and that routine experimentation is all that is required to produce, screen and select multiple strains having the recited genetic modification(s). I also agree with the Applicants when they state that the advent of recombinant technology cannot be used by the Examiner to dismiss classical techniques that have been used for years. I also agree with the Applicants' statement that once a beneficial mutation is discovered by genetic techniques including screening/selection, the sequence information can be determined and used,

if desired, to produce the same mutation using molecular biology, but that this prior knowledge is not necessary for one of skill in the art to make and use the present invention.

In my opinion, the screening described in Example 5 of 4368 transformants for glucosamine production is routine in the art and was accomplished in a single experiment. It resulted in the finding of 96 improved producers without any knowledge of the sequence of the enzyme or of the gene involved. Thirty of these 96 strains had the largest halos, and were rated as superior. Six strains were chosen from the superior 30 as the best producers of glucosamine. This simple procedure is predictable with regard to yielding strains meeting the requirements of improved glucosamine production. Of the 6 strains, three had the expected phenotype of reduced feedback inhibition and produced very high levels of glucosamine, as described in Example 6. The number 3 of improved mutant strains was high and such a result was predictable.

I further disagree with the Examiner when he states that the specification forces one skilled in the art to do trial and error experimentation to arrive at a glucosamine-6-phosphate synthase that has increased activity or decreased product inhibition. This is not "trial and error experimentation". It is experimentation following a carefully detailed procedure which yields a desired result.

To support my position, I cite and briefly discuss a number of recent scientific publications in which molecular biology techniques are accompanied by necessary screening/selection techniques.

Literature Cited

Q. Wang et al., Metabolic Engineering of *Torulopsis glabrata* for Improved Pyruvate Production. Enzyme and Microbial Technology 36:832-839, 2005. *T. glabrata* strains were metabolically engineered for increased production of pyruvate. First, *T. glabrata ura* strains (requiring uracil for growth) that were suitable for genetic transformation were isolated by ethyl methansulfonate mutagenesis, and selection by growth in media containing the antimetabolite 5-fluoroorotic acid. Then, the gene encoding PDC was specifically disrupted via homologous recombination with the *Saccharomyces cerevisiae URA3* gene as the selective marker. The disruptants displayed higher pyruvate production (20 grams/liter) and less of the undesirable ethanol production (4.6 grams/liter) than the parental strain (7-8 grams/liter pyruvate and 7.4 grams/liter ethanol). The disruptants

were able to produce in a 52 hour jar fermentation 82 grams/liter. It is clear that in this impressive work, selection was crucial for the process of strain improvement.

K. Stutzman-Engall et al., Semi-synthetic DNA Shuffling of *avaC* Leads to Improved Industrial Scale Production of Doramectin by *Streptomyces avermitilis*. Metabolic Engineering 7:27-37, 2005. The commercial Pfizer product Doramectin™ is made from the avermectin analog doramectin CHC-B1. This analog is made by fermentation with *Streptomyces avermitilis* along with the undesirable analog CHC-B2. To improve the ratio between the preferred CHC-B1 and the undesirable CHC-B2, the biosynthetic gene *avaC* was subjected to iterative rounds of semisynthetic DNA shuffling, a tool of molecular genetics. The resultant library of 5000 shuffled *aveC* variant strains was screened by fermentation in small plates containing 96 wells and assayed by high throughput mass spectrometry to determine ratios. Eighty nine strains were found to have improved ratios. The 89 were then fermented in flasks yielding 8 strains with the highest ratios. The procedure was repeated and the best strain was found to exhibit a 23-fold ratio improvement over the starting strain. This very important result was thus the product of the modern molecular genetic technique of gene shuffling combined with the necessary process of screening.

J. Mampel et al., Single-gene Knockout of a Novel Regulatory Element Confers Ethionine Resistance and Elevates Methionine Production in *Corynebacterium glutamicum*. Applied Microbiology and Biotechnology. 68:228-236, 2005. *C. glutamicum* was mutagenized by the recombinant DNA technique known as transposon mutagenesis. 7000 mutants were screened for rapid growth on agar plates containing the methionine antimetabolite known as DL-ethionine. Transfer of the ethionine resistance trait to another strain inactivated the *NCg12640* gene resulting in 2-fold overproduction of methionine as compared to the original strain. Thus, success was obtained using the genetic technique of transposon mutagenesis coupled to selection with ethionine and screening in flask fermentation.

G. Stephanopoulos et al., Exploiting Biological Complexity for Strain Improvement through Systems Biology. Nature Biotechnology 22:12612-1267, 2004. This review discusses the new concept of systems biology to improve strain improvement. The technology depends

on introducing a variety of perturbations and measuring the system response. They state that "a diverse set of tools has emerged to create gene deletions and amplifications which are used in conjunction with altered environments. Molecular biology advances have made it possible to perform these modifications at will. In addition to gene-specific techniques, several combinatorial tools have been developed, which, when combined with high-throughput screening, allow for randomized gene-expression levels...Advances in high-throughput assays greatly enhance our ability to characterize the cellular phenotype...Applying these tools to a microbial system provides a detailed snapshot of cellular function."

Z. Xu et al., A High-Throughput Method for Screening of Rapamycin-Producing Strains of *Streptomyces hygroscopicus* by Cultivation in 96-Well Microtiter Plates. Biotechnology Letters 27:1135-1140, 2005. The authors state that traditional mutation is still used to improve industrial strains and that new techniques such as genome shuffling still require positive mutants to be screened out from a large number of isolates obtained by recursive protoplast fusion. They further state that a variety of screening methods have been developed for different types of microorganisms including the "agar-plug" method and subsequent modified versions. In this paper, they describe a new screening method involving the culture of mutants on the surface of 96 agar-solidified wells in microtiter plates. By use of this screening technique, they were able to double the production titer of rapamycin.

W.J. Coleman et al., Solid-Phase High-Throughput Screening of Enzyme Variants: Detecting Enhanced Nitrilase Activity. Industrial Biotechnology 1:102-105, 2005. The authors discuss the modern technique of directed evolution of enzymes and state that it consists of the following steps: (1) mutating the gene encoding the enzyme to create a large population of variants; (2) expressing the gene products in a host organism; (3) screening for the desired properties; and (4) retrieving the desired variants. These steps can be repeated until the ideal enzyme variant is obtained. Their technique called Kcat technology monitors enzymatic activity of tens of thousands of enzyme variants simultaneously by acquiring full spectral and/or kinetic information from microcolonies that are simultaneously undergoing a color-forming reaction catalyzed by the enzyme that

the microbial colonies express. Each assay disk contains about 9000 individual microcolonies. Colonies with the highest activity are picked. Using such a screening method, over one million variants can be screened per instrument per day.

To summarize, let me say that that (1) one skilled in the art would not have to know the specific genetic modification(s) to perform on a specific gene or promoter or protein or enzyme to achieve an improved process; (2) routine experimentation in the art includes screening and/or selection; (3) such screening/selection is a necessary part of all strain improvement efforts, even when using the most modern genetic methods to cause mutations in the microbe; (4) such experimentation is not undue or unpredictable, but is simple, rapid, and will succeed provided that the method of screening/selection is clearly stated, as it has been by the Applicants; and (5) the Applicants have devised an elegant procedure to obtain industrially relevant concentrations of a medically useful molecule and deserve patent protection for the process.

6. I hereby declare that all statements made herein of my own are true and that all statements made on information and belief are believed to be true; and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the subject application or any patent issuing therefrom.

March 16, 2006
Date

Arnold L. Demain